Vegetable induced gene expression changes in anticarcinogenic pathways in human and mouse tissues

Chapter 7

Summary and general discussion

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Food preparation and dietary habits are very relevant exogenous factors affecting cancer risk. The most consistent finding on diet as a determinant of cancer risk is the association between consumption of vegetables and fruit and reduced risk of several cancers. Convincing epidemiological evidence exists for cancer of the gastrointestinal tract (oral cavity, pharynx, esophagus, stomach, colon and rectum) and of the respiratory tract (lung).[1] In terms of incidence, lung cancer is the most common cancer worldwide, closely followed by cancer of the colorectum as the third most frequent.[2] Diets containing considerable amounts of a variety of vegetables (> 400 g/day) may reduce these types of cancer by 45 respectively 40 percent (range 30-50).[3] The mechanisms by which vegetables exert their beneficial effects are various and studied in experimental systems. However, whether vegetables exert their effect by affecting genetic pathways is still mostly unknown, because only few studies have investigated the effect of vegetables and vegetable constituents on gene expression changes in target organs. Nowadays, microarray technology can be used to investigate the effect of a specific diet on the expression of multiple genes or proteins in a single experiment. It can help to identify the genetic mechanisms by which vegetables affect cancer risk at the molecular level.

The hypothesis investigated in the present thesis is that an important contribution of the anticarcinogenic effects of vegetables in the colorectum and lung is through modulating the expression of genes involved in biological and genetic pathways that are relevant for chemical carcinogenesis. Therefore, the aim is to provide more insight into the molecular mechanisms by which vegetables exert their anticarcinogenic effects, by focusing on the effects at the genome level in the relevant target cells.

The model of colorectal cancer (CRC) proposed by Fearon and Vogelstein consists of successive genetic changes, in which a number of genes are involved, including APC (adenopolyposis coli), K-RAS, DCC (deleted in colorectal cancer) and p53.[4] During the last decade, additional genetic events and specific molecular pathways have been identified. It became clear that the intact or mutated key molecules of the Vogelstein model interact and form a network of molecular events affecting additional genes.[5, 6] These genetic pathways and the involved genes are obvious molecular targets for the protection against CRC by vegetables. To identify the genes which are modulated *in vivo* in colorectal mucosa from humans by vegetables, the study described in **Chapter 2** was carried out. In this human dietary intervention study the effect of a 50% decreased (= 75 g/day) or doubled intake (= 300 g/day) of a mixture of vegetables (cauliflower, carrots, peas and onions) during two weeks on gene expression changes in normal colorectal mucosa of female sporadic adenoma patients and healthy controls was investigated by means of

microarray analysis of 597 genes representing pathways relevant for carcinogenesis. The two study groups were chosen to investigate whether the effect of the intervention is different for patients compared to controls, as it is plausible that vegetables exert a different effect in persons who are at higher risk for the development of CRC. In total, comparison of pre- and post-intervention rectal biopsy samples revealed that 52 genes were differentially expressed, and according to literature review, 20 of these genes are likely to be related to (colon)carcinogenesis. In both patients and controls, seven genes were similarly modulated, for example fos proto-oncogene and ornithine decarboxylase. Thirteen genes were modulated differently in patients compared to controls, including cyclooxygenase-2 and mdm2-A in patients and cytochrome P450-27B1, -2C19, -2D6, -2C9, and -3A4 in controls. An increased intake of vegetables resulted in down-regulation of genes promoting cell proliferation and bioactivation of procarcinogens, and in up-regulation of genes involved in cell growth arrest; in contrast, a decreased intake of vegetables resulted in down-regulation of genes inhibiting cell growth and up-regulation of genes promoting cellular dedifferentiation and bioactivation of procarcinogens. Furthermore, it seems that vegetables in patients affect genes involved in late stage of CRC, whereas in controls genes involved in the initiation phase are modulated. This is the first human study in which the effect of a vegetable intervention on gene expression changes at target level has been investigated. The results show that almost all the effects on modulating the expression of genes by altering vegetable intake can be mechanistically linked to cellular processes that explain either prevention of colorectal cancer risk by high vegetable intake or increased colorectal cancer risk by low vegetable intake.

To confirm the human findings and further elucidate mechanisms, mouse studies were performed using a comparable approach and technique. Although mice are often used to investigate the effect of diet on carcinogenesis[7-10], the number of studies in which the effect on gene expression is studied is limited, is mostly investigated in combination with carcinogen administration and is restricted to analyses of a few genes. Therefore, the mouse studies carried out in this thesis provide much information for the effect of specific vegetable diets on the expression of multiple biologically relevant genes in target tissues. To achieve this, a mouse cDNA microarray was constructed, which contained the same genes as present on the microarray used for the human study.

To mimic the human dietary intervention study, the mouse study illustrated in **Chapter 3** was performed in which the dose-dependent effect of a vegetable mixture was investigated on gene expression changes in colon mucosa. C57BL6 female mice were fed one of four different diets, containing no vegetables, 10%, 20% or 40% wt/wt vegetables mixture respectively, for a period of two weeks. The vegetables mixture consisted of the same combination and proportion of vegetables as used in the human intervention study, i.e. cauliflower (30% wet wt), carrots (30% wet wt), peas (30% wet wt), and onions (10% wet wt). The results of this study show a dose-dependent effect of vegetables on gene

expression, although in general this relationship is not linear. Almost all of the gene expression modulations occurred in the highest vegetable dose (40%) group. In total, the expressions of 39 genes were modulated, 17 of which are likely to play a role at different stages during CRC development, as indicated by literature review. Furthermore, for almost all these genes the altered expression can indeed explain reduced CRC risk. A diet high in vegetables positively modulated genes involved in different pathways, including inhibition of carcinogen formation, increasing DNA repair capacity, induction of apoptosis, and in reducing cell growth and tumor invasion. Many new genes were identified for which currently no modulation by vegetables has been reported, such as the detoxification gene ALDH1A1, several apoptosis genes (TNFRSF6, CASP3, -4 and -7, CTSB, TMSB10 and STAT1) and the tumor suppressor genes RRM1 and SLC26A3. Comparing the results of this mouse study with the results of the human study, only one gene is similarly affected by the vegetables in human and mouse colon, i.e. stearoyl-CoA desaturase (SCD). To date, two mouse (SCD1 and SCD2) genes and a single human SCD gene have been cloned and characterized. SCD is the rate-limiting enzyme in the cellular synthesis of monounsaturated fatty acids from saturated fatty acids. A proper ratio of saturated to unsaturated fatty acids contributes to membrane fluidity and cell-cell interaction. Abnormal alteration of this ratio has been shown to play a role in several physiological and disease states including diabetes, cardiovascular disease, obesity, hypertension, neurological disease, immune disorders, and cancer. However, causal relationships between SCD activity and these various disease states remain unclear. The activity of SCD is sensitive to for instance dietary changes, hormonal imbalance, temperature changes, alcohol and phenolic compounds.[11] SCD activity was decreased in rat liver during starvation and diabetes and was rapidly induced to high levels upon refeeding high carbohydrate diets or upon insulin administration.[12] The higher amount of carbohydrates present in the high vegetable diets could be responsible for the induction of SCD gene expression. The consequences of this on CRC risk are, however, indistinct. Although only one gene is similarly modulated in both studies, the results of both studies showed that a diet high in vegetables modulates genes in favor of CRC risk prevention via a number of similar pathways, i.e. inhibition of cell proliferation and detoxification of procarcinogens.

Despite the fact that gene expression studies are a fast and reproducible way to make an inventory of the effects of vegetables on gene expression changes, changes at the mRNA level are not necessarily proportional to the changes at the protein level because of differences in protein translation and degradation. Furthermore, proteins can undergo several functional posttranslational modifications like phosphorylation and glycosilation. Therefore, in addition to the gene expression analysis, a proteomic approach was undertaken to evaluate the effect of different doses of the vegetable mixtures on proteome changes in colon mucosa of female C57BL6 mice (**Chapter 4**). Differential protein expression was determined with 2D gel electrophoresis. Thirty proteins were found to be

differentially expressed, and six proteins could be identified by mass spectrometry, namely MLRN, CAH-1, HMG-1, PAP3, GAPDH and OSCP. Except for OSCP, alterations in the levels of these proteins coincide with a pivotal role in the protection against colon cancer. The proteins are involved in different processes like cell growth, cell differentiation and apoptosis.

Comparing the results from the proteomic study with the gene expression study, no correspondence was observed between the human gene colon study and the mouse proteomic colon study. Only GAPDH was found to be differentially expressed in both the mouse proteomic study as in the mouse gene expression study, although dissimilar. Protein analysis by 2D-gelelctrophoresis indicated a decreased protein expression in all diets compared to control, although this was not statistically significant. The immunoblotting results on the other hand showed a large variation between the various sub-pool samples. The observed difference between the mRNA levels and protein levels may be due to different subforms of GAPDH. To exert the wide variety of GAPDH functions, the different subforms of the protein need to be distinguished by the cell. This might be regulated by alternate splicing or posttranslational modification and results in protein products that differ in isoelectric point (pI) and/or molecular weight. This might also explain the difference in protein expression as observed from the 2D-gelelectrophoresis and the immunoblotting experiments.[13, 14] Considering the role of GAPDH in DNA replication, DNA repair and apoptosis, GAPDH might be an interesting candidate for protection against colon cancer.[14] However, this has to be further investigated. The cDNAs representing the mRNAs coding for the other proteins found in the proteomic study were not present on the microarray used in the gene expression studies. However, besides GAPDH, for none of the other genes present on the array, differentially expressed proteins were identified. It could be that the gene expression difference resulted in a protein difference too small to be identified in the proteomics analysis. Despite this, in addition to the gene expression results, the results of the proteomic study also indicate modulation of proteins in favor of prevention of CRC risk.

Next to the amount of vegetables, the type of vegetables can be of importance for inducing particular gene expression changes. Therefore, the study described in **Chapter 5** was carried out in which the effect of four individual vegetables, i.e. cauliflower, carrots, peas and onions, on the expression of genes in colon mucosa of female C57BL6 mice was examined. In total, 18 genes were differentially expressed by one or more of the specific vegetables: cauliflower was able to modulate most genes (10), closely followed by the onions (7) and carrots (7); least gene expression modulations occurred in the peas group (3). No genes were modulated solely by the carrots. According to literature review, ten of the 18 modulated genes are likely to be involved in CRC development, and seven out of these 10 are modulated in favor of CRC prevention. The four individual vegetables have about the same potential in modulating genes in support of lower CRC risk, although

mostly via different mechanisms. Genes involved in cell growth regulation and maintaining homeostasis were affected in all vegetable groups. Furthermore, cauliflower, carrots and peas affected genes involved in induction of apoptosis; onions modulated genes which could play a role in maintaining DNA stability and induction of cellular differentiation; and peas, in addition to increasing the expression of an apoptosis gene, induced an angiogenesis inhibiting gene. From epidemiological studies it is known that with respect to prevention of CRC especially cruciferous and green leafy vegetables play a role.[1] Indeed, cruciferous vegetables were able to induce most gene expression differences; however, most of these genes are currently not known to be involved in CRC protective mechanisms. The contribution of carrots, peas and onions in prevention of CRC risk could be of greater importance than until now considered.

In addition to this evaluation of vegetable specific gene modulations, a comparison can be made with the results of the studies described in **Chapters 2 and 3**. First, an evaluation will be made with the mouse vegetable mixture study described in **Chapter 3**. The amount of the individual vegetables is equal to the amount of these specific vegetables in the highest vegetable mixture group (40%) and therefore the contribution of the four individual vegetables to the combined effect of these vegetables on gene expression can be investigated. Eleven similar genes were modulated in both the vegetable mixture study and the individual vegetable study and for seven of them the modulation by the mixture could be explained by the effect of a particular vegetable. ACTB and CASP4 were modulated in the cauliflower group; OAT, EFHU1 and PMP22 were affected by cauliflower and carrots; and SCD2 and HIF1A were modulated by the onions. Although most of these genes (i.e. ACTB, EFHU1, PMP22 and SCD2) are at this time not known to be involved in CRC protective mechanisms, the modulation in both vegetables studies gives a strong indication of their importance. Their role, however, in CRC development has to be further investigated. Comparing the results of the individual vegetables study with the human study illustrated in **Chapter 2**, two genes were similarly affected, i.e. ODC and, again, SCD. The role of SCD has already been discussed. The fact that modulation of this gene was observed in three independent vegetable studies and that the modulation is found in mice as well as in humans, indicates a strong regulation by vegetables and makes this gene of particular interest for further research. In addition to SCD, ODC was similarly modulated in the human study compared to the mouse individual vegetable study. This gene was downregulated in the high vegetable diet groups in both patients and controls, and in the mouse cauliflower and carrots group. ODC catalyzes the first step in the polyamine biosynthetic pathway, a highly regulated pathway associated with rapid growth states, including tumorigenesis. The polyamines are ubiquitous cellular polycations essential for optimal rates of cell growth and –differentiation. Increases in intracellular polyamine levels are related to increased cell proliferation and higher CRC risk.[15, 16] In normal colonic epithelium, polyamine levels are maintained at low levels, coordinated by the tumor

suppressor gene APC. APC mediates ODC expression via a C-MYC dependent mechanism affecting ODC transcription. Wild-type APC suppresses the level of β-catenin, thereby reducing the formation of a complex between β-catenin and the lymphoid-enhancing factor (LEF)/T-cell factor (Tcf) resulting in reduced expression of target genes like C-MYC and subsequent C-MYC target genes, like ODC.[16] Inhibition of ODC by vegetable components has already been reported for β-carotene[17], present in carrots, and indole-3-carbinol[18], present in cauliflower. The results of the studies described in this thesis (**Chapter 2** and **4**) are in line with these previous investigations and provide more evidence of regulation of ODC expression by vegetables.

The polyamine biosynthetic pathway plays an important role in cell growth and differentiation in the intestine. Two other genes involved in this pathway were modulated in the vegetable mixtures study (**Chapter 3**), i.e. OAT and SAT; OAT was also affected in the cauliflower and carrots group (**Chapter 5**). OAT catalyzes the conversion of ornithine, which is the substrate for ODC, to glutamate semialdehyde, thereby reducing intracellular ornithine contents.[19] Both in the highest vegetable mixture group (40%) as in the cauliflower and carrots group, the expression of OAT was downregulated, which could result in an increased ornithine available for ODC, leading to higher polyamine levels. However, SAT, a key gene involved in polyamine catabolism, was upregulated in the highest vegetable group (40%). This gene encodes for the key enzyme catalyzing the formation of shorther-chain amines from longer-chain ones, thereby reducing polyamine contents. The expression of the SAT gene is regulated by the K-RAS gene, which is an important proto-oncogene involved in development of CRC. Mutated K-RAS leads to a suppression of the nuclear hormone receptor peroxisomal proliferators-activated receptor γ (PPARγ), which in turn leads to a decreased SAT expression.[16] The modulation of ODC, OAT and SAT by the vegetables provides evidence for vegetables to exert part of their beneficial effects in CRC through affecting the polyamine biosynthetic pathway, leading to reduced cell proliferation.

In addition to prevention of CRC, epidemiological studies provide evidence for protection against lung cancer incidence by vegetables and fruits. Particularly dark green and yellow-orange vegetables, rich in β-carotene and vitamin E, are the specific types of vegetables, which best protect against lung cancer. In contrast to the colon and rectum, the lungs are not a site of direct contact, i.e. the vegetables pass the gastrointestinal tract but not the lungs as they are ingested. Furthermore, the dietary compounds that reach the lung may have been modulated by the first-pass metabolism in the liver. Therefore, the lungs are subject to only systemic exposure of vegetables-derived compounds and the mechanisms by which vegetables protect against lung cancer could therefore be different compared to that in the colon. To investigate the effect of vegetables on gene expression in the lung, the lungs of the mice used in the studies described in **Chapters 3** and **5**, were analyzed for gene expression changes (**Chapter 6**). In the vegetable mixture groups, 18

genes were differentially expressed. Similar as in the colon study (**Chapter 3**), most gene expression differences occurred between the highest vegetable dose group (40%) and one of the other diet groups. However, only seven of the 18 genes are, according to literature review, likely to be involved in mechanisms underlying lung cancer risk prevention. But, the hypothesized effects of gene expression modulation by the vegetable mixture are not always in favor of lung cancer risk prevention. The effects on IGFBP3 (↓), TGM2 (↓), TOP2A (↑), GLUL (↓) and CTSS (↓) are expected to enhance lung cancer risk, whereas the effects on GAPDH (↑) and FCER1G (↑) are expected to result in lung cancer risk prevention. In the individual vegetable groups, 11 genes were modulated, eight of which might be implicated in lung cancer preventive mechanisms, as indicated by literature review. Most gene modulations (6) took place in the carrots group, followed by peas (3) and cauliflower (2). Onions, together with carrots, were able to modulate the expression of only one gene, i.e. CTSS. The effect, however, on the expression of this gene is expected to increase lung cancer risk, by suppression of the immune system. Furthermore, also for the other modulated genes, the theoretical effects of the modulation by the individual vegetables on mechanisms underlying lung cancer prevention are not always beneficial. With respect to the effect of carrots on gene expression changes, five out of six modulated genes could be involved in lung cancer protective mechanisms; three (GLUL, SULT1A1 and SELENBP1) out of these five are affected in such a way that lung cancer protective mechanisms could be expected. For peas, two out of three modulated genes could be involved in lung cancer preventive pathways; only the effect on GSR could lead to lung cancer risk prevention by inducing the anti-oxidative defense. Finally, cauliflower was able to modulate the expression of two genes, i.e. BNIP1 (↑) and G1P2 (↓). Only BNIP1 is likely to be involved in lung cancer preventive pathways. Upregulation of BNIP1 by cauliflower could lead to increased apoptosis, which is generally regarded as a protective mechanism against cancer by removing genetically damaged cells before they can undergo clonal expansion. Taken all this together, vegetables are able to modulate genes involved in lung cancer preventive pathways, although, the modulation is not unambiguous in favor of reduced lung cancer risk. It is difficult, however, to predict the contribution of each gene modulation in the cancer process. The net effect of the gene expression modulation by the vegetables could still result in lung cancer risk prevention. Furthermore, genes which were not present on the microarray could play a role in lung carcinogenesis. More research is needed to investigate the effect of vegetables on gene expression changes in the lungs. Very interestingly, carrots were able to modulate most gene expression changes. Taken into account that it is known from epidemiological studies that in particular consumption of high amounts of carrots are able to protect against lung cancer, the results of the lung study provide rationale that indeed carrots are of importance in modulating this process.

Comparing the gene expression modulation occurring in colon with those in lung, corresponding and different genes were affected. Genes which are modulated in both

tissues provide evidence for similar mechanisms by which vegetables protect against both types of cancer. When comparing the mouse lung study with the mouse colon study (**Chapter 3, 4 and 5**), eight genes were modulated in both the mouse colon and lung and include EFHU1, G1P2, GAPDH, SULT1A1, SEPP1, HPGD, TOP2A and BNIP1, however, the modulation was opposite (except for GAPDH). As already mentioned, the dietary compounds that reach the lung may have been modulated by the first-pass metabolism in the liver and these metabolites could be responsible for the observed effects. No (other) explanation for this remarkable result can be given and this has to be further investigated. The role of EFHU1, G1P2, and SEPP1 in carcinogenesis is currently not known. GAPDH (also identified in the colon proteomic study as differentially expressed, **Chapter 4**) and BNIP1 are involved in apoptosis, TOP2A plays a role in DNA replication, and SULT1A1 and HPGD are involved in biotransformation. In addition to modulating of similar genes, genes were affected by vegetables in either colon or lung. The number of genes affected in lung tissue is smaller and no genes are overrepresented in mechanisms of cancer prevention. However, remarkably, carrots seem to have the greatest potential of modulating genes in the lung, whereas in colon the individual vegetables appear to have equal promise for modulating genes in favor of CRC prevention.

Overall, the studies presented in this thesis provide much information on vegetable induced gene expression changes in target tissue where cancer risk is reduced by increased vegetable intake. In particular, the studies regarding vegetable induced gene expression changes in the colon and rectum support the hypothesis investigated in this thesis. In colon, vegetables modulated a lot of genes involved in biological and genetic pathways in favor of prevention of CRC risk. Genes were modulated for which already a relationship with vegetables and/or cancer prevention has been reported. In this regard, the studies provide new evidence which strengthens this relationship. In addition to these 'known' genes, new genes were identified for which, until now, no modulation by vegetables has been described, and/or no link is known with cancer. Remarkably, the dose of vegetables necessary to exert a measurable effect on the expression of genes in favor of cancer preventive mechanisms is relatively high in mice. The evidence regarding lung cancer risk prevention by vegetable induced gene expression changes is less clear compared to the results of the colon studies. The role of carrots is of particular interest and most promising for modulating genes in support of reduced lung cancer risk. The results of the lung studies, however, provide too little information to either reject or confirm the hypothesis. Further research is needed in which the contribution of specific genes and pathways modulated by different vegetables types is investigated. Several genes are of particular interest for future studies. Especially SCD is of interest because the modulation by vegetables was observed in three independent vegetable studies in mice as well as in humans. In addition, the role of COX-2 mediated gene expression by vegetables is very

relevant, because of its established role in CRC risk. In addition to specific genes, several pathways are of particular interest. In both human and mouse studies, mostly genes involved in inhibition of cell growth are modulated by the vegetables. The affected pathways predominantly comprise the polyamine biosynthetic pathway and the apoptosis pathway. By using knockout and/or transgenic systems, the effect of vegetables on the contribution of the absence or presence of particular genes in the cancer process can be examined. Relatively new technologies like RNA interference and laser capture microdissection provide new possibilities in optimizing gene expression research. Furthermore, to investigate the effect of vegetables on gene expression changes in different stages of the carcinogenic process, studies in which vegetable are applied before and/or after administration of carcinogens can be carried out. In addition to dose-response studies, time-line studies could provide information about specific time-points and duration of vegetable induced gene expression effects. The results of these studies will provide a more complete and fundamental understanding of cancer aetiology, which is necessary for the development of improved prevention strategies based on dietary advice. Improved and more convincing evidence for the anticarcinogenic effects of vegetables may result in higher consumption of vegetables by the general public.